



# Adverse effects in lumbar spinal cord morphology and tissue biochemistry in Sprague Dawley male rats following exposure to a continuous 1-h a day 900-MHz electromagnetic field throughout adolescence

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## ABSTRACT

Cell phones, an indispensable element of daily life, are today used at almost addictive levels by adolescents. Adolescents are therefore becoming increasingly exposed to the effect of the electromagnetic field (EMF) emitted by cell phones. The purpose of this study was to investigate the effect of exposure to a 900-MHz EMF throughout adolescence on the lumbar spinal cord using histopathological, immunohistochemical and biochemical techniques. Twenty-four Sprague Dawley (28.3–43.9 g) aged 21 days were included in the study. These were divided equally into three groups – control (CG), sham (SG) and electromagnetic (ELMAG). No procedure was performed on the CG rats until the end of the study. SG and ELMAG rats were kept inside an EMF cage (EMFC) for 1 h a day every day at the same time between postnatal days 22 and 60. During this time, ELMAG rats were exposed to the effect of a 900-MHz EMF, while the SG rats were kept in the EMFC without being exposed to EMF. At the end of the study, the lumbar regions of the spinal cords of all rats in all groups were extracted. Half of each extracted tissue was stored at  $-80^{\circ}\text{C}$  for biochemical analysis, while the other half was used for histopathological and immunohistochemical analyses. In terms of histopathology, a lumbar spinal cord with normal morphology was observed in the other groups, while morphological irregularity in gray matter, increased vacuolization and infiltration of white matter into gray matter were pronounced in the ELMAG rats. The cytoplasm of some neurons in the gray matter was shrunken and stained dark, and vacuoles were observed in the cytoplasm. The apoptotic index of glia cells and neurons were significantly higher in ELMAG compared to the other groups. Biochemical analysis revealed a significantly increased MDA value in ELMAG compared to CG, while SOD and GSH levels decreased significantly. In conclusion, our study results suggest that continuous exposure to a 900-MHz EMF for 1 h a day through all stages of adolescence can result in impairments at both morphological and biochemical levels in the lumbar region spinal cords of Sprague Dawley rats.

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## 1. Introduction

Although the telephone has changed in shape and size over the years, it has become and remains an inseparable means of communication and part of daily life. While the use of land lines

has decreased with advances in technology, the use of wireless cell phones is increasing all the time. However, the growing use of cell phones, which are popular with all age groups, also causes a number of problems. While adults use cell phones more for work purposes, they have become toys that children and young people are reluctant to be parted from even for a moment (Bianchi and Phillips, 2005; Nikhita et al., 2015). Indeed, half of adolescents even take their cell phones with them to bed (Adachi-Mejia et al., 2014), and college students spend almost 9 h a day on their mobiles

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(Roberts et al., 2014). The increasing use of cell phones under the influence of attractive advertising campaigns and falling prices has led to their becoming the main source of exposure to electromagnetic field (EMF) in children, teenagers and adolescents (Kheifets et al., 2005).

The World Health Organization defines adolescence as the intermediate period between 10 and 19 years when the individual is no longer a child but not yet fully adult (World Health Organization, 2016). It is a transitional period when individuals diversify and mature both physiologically and neurologically and progress from childhood to adulthood (Spear, 2000). As with many systems in the human body, the central nervous system (CNS) that begins developing in the intrauterine period continues to do so after the postnatal (PN) period and throughout adolescence. Cortical and spinal maturation in the nervous system continues until adulthood (Smith et al., 2015; Fietzek et al., 2000). A large number of clinical and experimental studies have therefore investigated the effects of various environmental factors on the CNS in adolescence (Smith et al., 2015; Risher et al., 2015; Weiss et al., 2015; Mitchell et al., 2014). This is particularly because any agent that might impact on normal CNS development during this time may also lead to consequences that affect the rest of life. Although there have been several studies on agents that may cause changes in the CNS in the PN period, the number of studies investigating the effects of exposure to EMF in adolescence is quite limited. Yet EMF emitted by numerous electronic devices, including cell phones, that are overused during adolescence causes oxidative stress and injury in several tissue, such as the liver, heart and testis (Topal et al., 2015; Hancı et al., 2013; Türedi et al., 2015). Rat studies have reported that EMF causes injury leading to oxidative stress in nervous system tissues (İkinci et al., 2016). In addition, the application of 900-MHz EMF has been shown to cause a decrease in neurons in the hippocampus of both adult rats and of pups born to pregnant rats (Bas et al., 2009; Odaci et al., 2008). There are also studies reporting that EMF causes injury in cultured neurons, as well as impairing the blood brain barrier and leading to neuronal degeneration in the brain (Xu et al., 2010; Nittby et al., 2009).

Some authors have reported that the spinal cord can act as an antenna for the effects produced by EMF in the CNS (Balaguru et al., 2012). There is therefore a strong probability that the spinal cord, a component of the CNS that enables constant communication between the brain and peripheral nervous system will also be affected by EMF. Indeed, Odaci et al. (2013) reported impairment in motor function tests and pathological changes in the spinal cord in rat pups exposed to EMF during pregnancy. However, we encountered no studies in the literature investigating the potential effects on the spinal cord of exposure to EMF throughout adolescence.

It has already been established that investigation may provide useful data on the possible adverse effects of 900-MHz EMF on the human child spinal cord since the age of 8–9 weeks in Sprague Dawley rats is comparable to human preadolescence (Tirelli et al., 2003; Şahin et al., 2015). The purpose of this study was therefore to use histological and biochemical methods to investigate changes that may occur in the lumbar region of the spinal cord in male rats following daily 1-h exposure to a 900-MHz throughout adolescence between PN days 21 and 60.

## 2. Material and methods

### 2.1. Animals and experimental procedures

The study was performed following approval from the Karadeniz Technical University (KTU) Animal Care and Ethics Committee, Turkey. Twenty-four Sprague Dawley rats aged 21 days

and weighing 28.3–43.9 g obtained from the KTU Surgical Research Center were used. These were kept in standard plastic rat cages in the laboratory (room temperature  $22 \pm 2^\circ\text{C}$ , humidity 50% in a 12-light and 12-h dark cycle) and given standard rat chow and tap water throughout the study. Rats were weighed at the beginning of the study and randomly divided into three equal groups. No procedure was performed on the control group (CG) rats until the end of the study. The sham (SG) and EMF group (ELMAG) rats were placed inside an EMF cage (EMFC) for 1 h a day at the same time every day from PN day to 22 until PN day 60 (SG at 10–11 a.m. and ELMAG at 11–12 a.m.). During this time, the ELMAG rats were exposed to the effect of a 900-MHz EMF, while SG rats were kept in the EMFC but were not exposed to EMF. At the end of the study (on PN day 60), rats from all groups were sacrificed under deep anesthesia (Ketalar® 50 mg/kg, Eczacıbaşı, Turkey). The lumbar region of the spinal cord of all rats was removed. Half of each tissue was stored at  $-80^\circ\text{C}$  for biochemical analysis, while the other half was placed in 10% formaldehyde for histopathological investigation. The animals were obtained from the KTU Surgery Research Center, where they were housed during the study and where the tissues were removed.

### 2.2. EMF application system and EMF exposure

A system we describe as the EMF application system was used for the application of EMF to the ELMAG rats. The EMF application system and the EMFC within it were as described in various previous studies of ours, where all details are available (Türedi et al., 2015; Topal et al., 2015; Odacı and Özyılmaz, 2015). In brief, the cage used in the system and known as the EMFC was made out of Plexiglas and measured  $30\text{ cm} \times 42\text{ cm} \times 52\text{ cm}$ . A half-wave dipole antenna made out of a 1-mm thick copper rod was inserted to a depth of 11 cm from the center of the open top of the EMFC. The antenna was attached to an ultrahigh-frequency oscillator (1218-BV, Lockable Oscillator, 900–2000 MHz, General Radio Company, Concord, MA, Serial no. 1483) set to an output power of approximately 300 mW and a frequency of 900 MHz via a coaxial cable. The oscillator was attached to an uninterrupted power source (1267-B Regulated Power Supply, General Radio Company, serial no. 903) in order to avoid electrical disruptions. The EMF application system was used in order to establish a 900-MHz EMF effect inside the EMFC during EMF application. EMF intensities inside and outside the EMFC were measured using a broadband EMF intensity measuring device (100 kHz–2.5 GHz range, Chauvin-Arnoux C.A 43 Fieldmeter, Chauvin-Arnoux Group, Paris, France) with a range of 100 kHz to 2.5 GHz. Measurements were performed at nine different points on the floor of the EMFC, from both inside and outside the cage, and with the EMFC empty and when it contained rats. These measurements confirmed an uninterrupted 900-MHz EMF effect inside the cage during EMF application.

### 2.3. Histological staining

Spinal cords set aside for histological staining were decalcified following 1-day fixation in 10% formaldehyde. Tissues were next washed in running water and passed through alcohol series and then made transparent with xylene before finally being embedded in paraffin blocks. For histopathological investigation, 5- $\mu\text{m}$  sections were prepared with the help of a fully automatic microtome (Leica RM 2255, Leica Instruments, Nussloch, Germany) and stained with cresyl violet. The sections obtained were analyzed under a light microscope (Olympus BX51; Olympus Co., Tokyo, Japan), and photographs were taken with a digital camera (Olympus DP 71 Olympus Co., Japan) attached to the same microscope.

## 2.4. TUNEL procedures

The terminal deoxynucleotidyl transferase (TdT) deoxyuridine triphosphate nick end labeling assay (TUNEL) method was used for immunohistochemical evaluation. The 5- $\mu\text{m}$  sections taken from the paraffin blocks were stained in accordance with the manufacturer's (In Situ Cell Death Detection Kit POD, catalog number: 11 684 817 910, Roche, Mannheim, Germany) instructions. Cells in gray matter in the TUNEL-stained sections were examined under a light microscope at a magnification of  $\times 400$  (Olympus BX51; Olympus Co., Tokyo, Japan). TUNEL (+) neurons and glial cells staining brown were identified as apoptotic. Five different fields were randomly selected in gray matter in each section. Normal and apoptotic neurons and glial cells were counted separately, and an apoptotic index (AI) was calculated for both neurons and glial cells ( $\text{AI} = \text{TUNEL (+) cell number} / \text{total cell number} \times 100$ ) (Türedi et al., 2015; Odacı and Özyılmaz, 2015). Use was made of Liu et al. (1997)'s study in the differentiation of apoptotic neurons and apoptotic glial cells in our study. Liu et al. (1997) reported that the nuclear diameters of apoptotic neurons were approximately half those of healthy neurons, and that

apoptosis caused an almost identical level of shrinkage in glial cell nuclei. Differentiation of apoptotic glial cells and neurons in our study was therefore performed by comparing the dimensions of cytoplasm and nuclei of apoptotic cells among themselves and with healthy neighboring neurons and glial cells.

## 2.5. Biochemical analyses

Glutathione (GSH), lipid peroxidation (LPO) concentrations, catalase (CAT) and superoxide dismutase (SOD) enzyme activities were measured in the spinal cord specimens stored at  $-80^\circ\text{C}$  for biochemical analyses. GSH levels in tissues were determined using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) according to the modified Sedlak and Lindsay (1968) method. The results were expressed as nmol/mg of tissue. LPO concentrations were calculated using the thiobarbituric acid test (Ohkawa et al., 1979) and tissues' malondialdehyde (MDA) levels. Quantities were expressed as nmol/mg of tissue. CAT activity was measured using the method described by Aebi (1984), the decrease in absorbance resulting from the breakdown of  $\text{H}_2\text{O}_2$  and its elimination was observed at 240 nm, and the results were expressed as mmol/min/

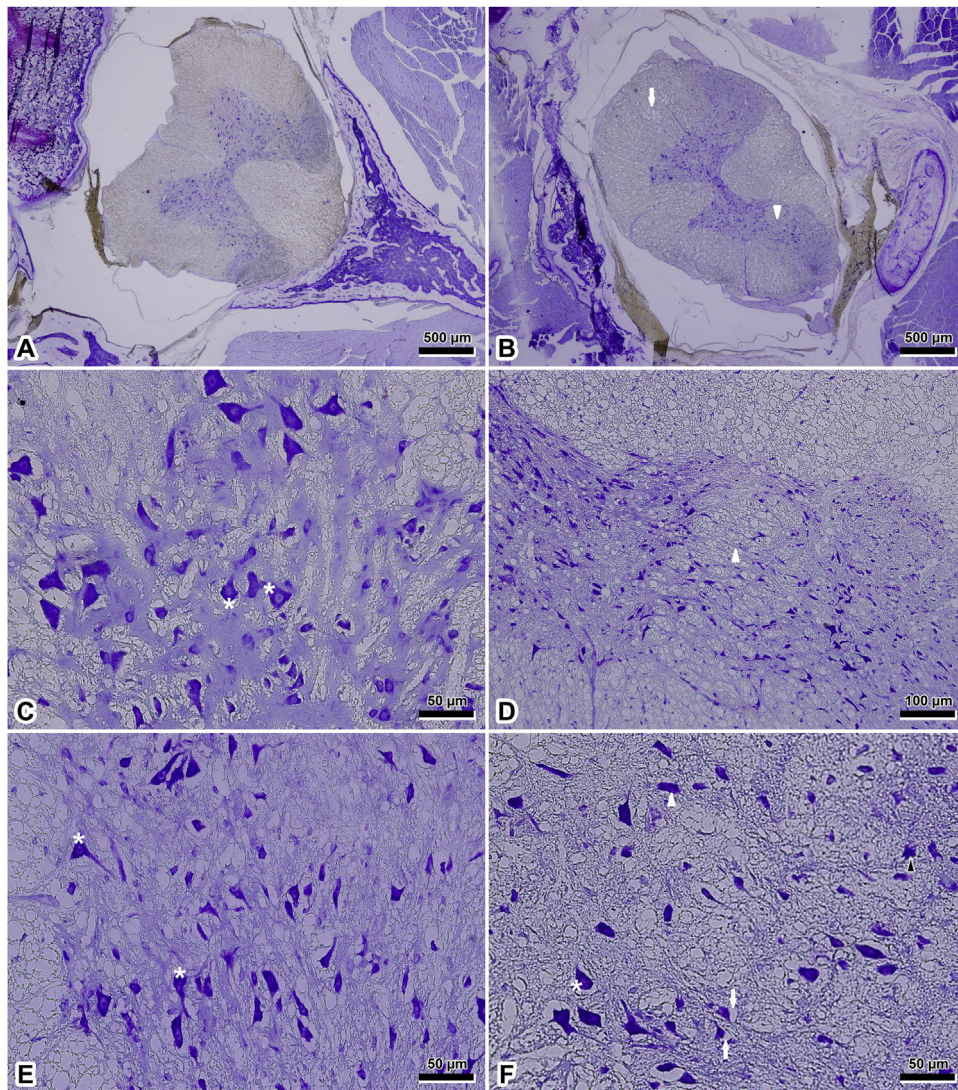


**Fig. 1.** While a normal gray and white matter structure were observed in the control group (A), irregular areas in gray matter, infiltration of white matter into gray matter ( $\blacktriangle$ ) and vacuolar regions in white matter ( $\triangle$ ) were present in the EMF group (B, D) (Cresyl violet, A, B  $\times 40$  and D  $\times 200$ ). Neurons (\*) with a euchromatic nucleus and normal morphology were present in gray matter in spinal cord from the control (C) and sham (E) groups, while in addition to neurons with a normal morphological structure (\*), occasional shrunken and degenerated neurons ( $\blacktriangle$ ) and vacuolization in neuron cytoplasm ( $\triangle$ ) were observed in the EMF group (F) (Cresyl violet,  $\times 400$ ).

mg of tissue. SOD enzyme values were determined using the method described by Sun et al. (1988). This method is based on the reduction of nitroblue tetrazolium by the superoxide producer xanthine – xanthine oxidase. SOD activity was measured at 560 nm and expressed as mmol/min/mg of tissue.

## 2.6. Statistical analysis

Kruskal–Wallis analysis of variance was used to compare differences between group parameters. Dual comparisons between groups exhibiting significant values were analyzed using the Mann–Whitney *U* test with Bonferroni correction. A difference at  $p < 0.01$  was considered statistically significant. Statistical analyses were performed using SPSS 13.1 (Statistical Package for the Social Sciences, version 13.1, SSPS Inc., Chicago, IL). Data were presented as mean  $\pm$  standard deviation.

## 3. Results

### 3.1. Light microscopy observations

Light microscopic examination of sections stained with cresyl violet revealed gray and white matter with a normal appearance in both CG and SG. Neurons and glia cells with a normal appearance and a centrally located round, euchromatic nuclei were observed in the gray matter in both groups (Fig. 1A, D and E). In ELMAG, however, there was pronounced irregularity in gray matter morphology, increased vacuolization, and white matter infiltration into gray matter. Shrunken cytoplasm was observed in some neurons in gray matter, while others stained dark. Intracytoplasmic vacuoles were also prominent in part of the cytoplasm (Fig. 1B, C and F).

### 3.2. Immunohistochemical results

Evaluation of TUNEL-stained sections revealed a statistically significantly higher apoptotic index in ELMAG glia cells compared to both CG and SG ( $p < 0.01$ ). The neuronal apoptotic index was also significantly higher in ELMAG than in CG or SG ( $p < 0.01$ ) (Fig. 2A–C) (Table 1).

### 3.3. Biochemical results

Statistical analysis of the data obtained from biochemical evaluation revealed a significant increase in MDA values in ELMAG compared to CG ( $p < 0.01$ ). ELMAG tissue SOD and GSH levels were significantly lower than those of CG ( $p < 0.01$ ) (Table 1).

## 4. Discussion

The spinal cord is the structure through which information is transmitted between the brain and the peripheral nervous system. Therefore, any change in the structure of the spinal cord may lead to compromise of communication between the brain and the peripheral nervous system (Odaci et al., 2013). Similarly to other components of the nervous system, a range of factors can affect the spinal cord, and its normal morphology may be impaired by numerous external and internal environmental agents, resulting in a high probability of functional impairment. Several studies have investigated the effects of EMF on CNS tissues (such as the brain, cerebellum and hippocampus) (Odaci et al., 2008, 2010; Odaci et al., 2013; Sonmez et al., 2010; Şahin et al., 2015). These have reported significant evidence for neuronal damage in such organs as the cortex, hippocampus and basal ganglia in the brains of rats exposed to EMF. This evidence includes both diffuse and clustered dark neurons, also often shrunken and dark stained, homogenized

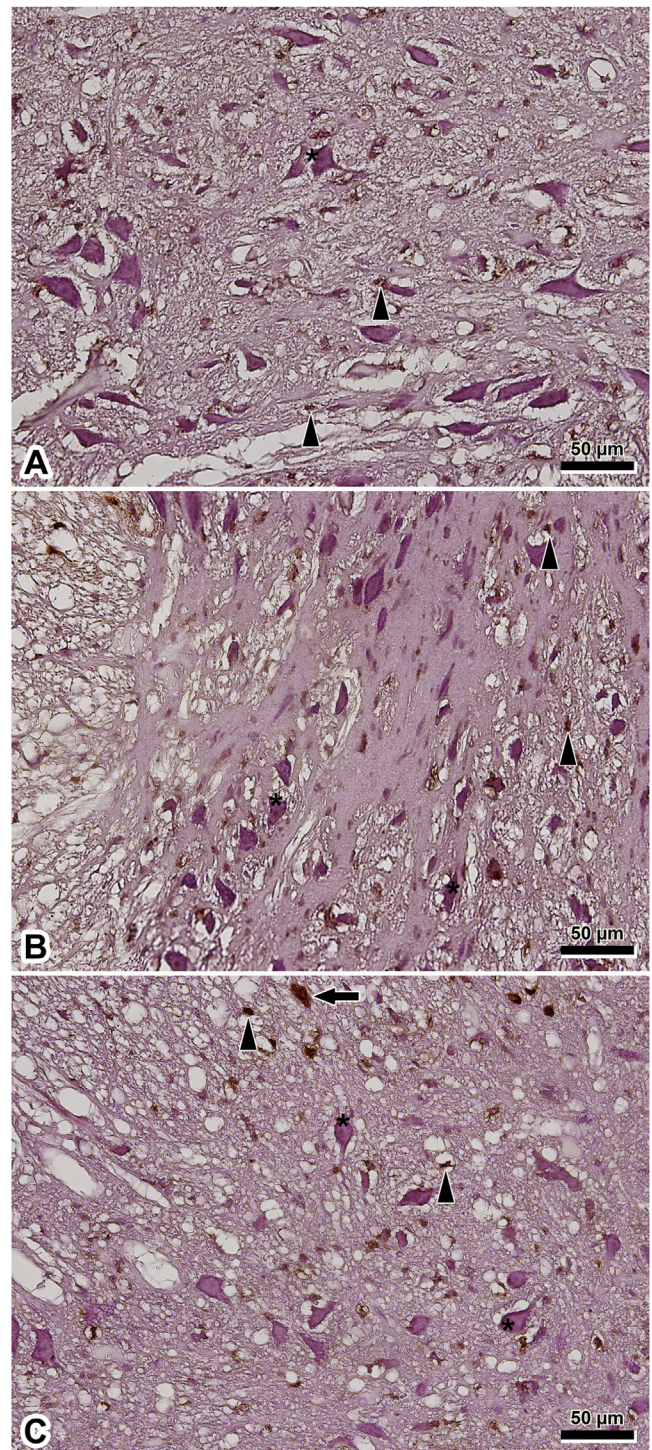


**Fig. 2.** TUNEL (–) neurons with a normal structure (\*) and a very few TUNEL (+) apoptotic glial cells (▲) were observed in sections from the control (A) and sham (B) groups, but no apoptotic neurons were identified. In the EMF group (C), however, in addition to neurons with a normal morphological structure (\*), a very few TUNEL (+) apoptotic neurons (†) and numerous apoptotic glial cells (▲) were identified (TUNEL  $\times 400$ ).

with compromise of visible internal cell structures at cresyl violet staining (Salford et al., 2003).

However, we encountered no previous studies using histological, immunohistochemical and biochemical techniques to investigate changes in the spinal cords of rats exposed to the effect of 900-MHz EMF during all stages of adolescence. This was the most

**Table 1**

Mean values for spinal cord tissue biochemistry and apoptotic index parameters of neuron and glial cells in the study groups.

| Parameters                   | CG            | SG            | ELMAG                     |
|------------------------------|---------------|---------------|---------------------------|
| MDA (nmol/mg tissue)         | 14.66 ± 0.50  | 16.17 ± 1.04  | 15.99 <sup>a</sup> ± 0.82 |
| SOD (mmol/min/mg tissue)     | 0.10 ± 0.01   | 0.07 ± 0.02   | 0.02 <sup>b</sup> ± 0.01  |
| CAT (mmol/min/mg tissue)     | 81.22 ± 11.32 | 87.42 ± 15.78 | 89.11 ± 7.31              |
| GSH (nmol/mg tissue)         | 0.74 ± 0.01   | 0.70 ± 0.06   | 0.69 <sup>b</sup> ± 0.01  |
| Neuronal apoptotic index (%) | 0.00 ± 0.00   | 0.25 ± 0.46   | 3.50 <sup>c</sup> ± 0.92  |
| Glial apoptotic index (%)    | 11.13 ± 2.9   | 15.13 ± 5.93  | 26.63 <sup>c</sup> ± 5.85 |

Data are expressed as mean ± SD. MDA, Malondialdehyde; SOD, Superoxide dismutase; GSH, Glutathione; CAT, Catalase; CG, control group; SG, Sham group; ELMAG, electromagnetic group.

<sup>a</sup> The ELMAG MDA level was significantly higher than that in CG ( $p < 0.01$ ).

<sup>b</sup> The ELMAG SOD and GSH levels were significantly lower than those in CG ( $p < 0.01$ ).

<sup>c</sup> The ELMAG neuronal apoptotic index and glial apoptotic index were significantly higher than those in CG and SG ( $p < 0.01$ ;  $p < 0.01$ , respectively).

important reason for the planning of this study. In one previous study, pregnant rats were administered 900-MHz EMF on a 1-hourly basis between days 13 and 21 of pregnancy, inclusive. The spinal cords of the pups born to these mothers were then examined using histopathological methods on PN day 32. The authors described morphological damage and atrophy in the spinal cord, vacuolization in gray matter accompanied by occasional myelin thickening, white matter infiltration among nerve fibers, loss of marginal distinction between white and gray matter and gray matter infiltration into white matter (Odaci et al., 2013). Structural compromises of the motor neuron pericaryon were also observed in the EMFG spinal cords of pups. Similarly to these findings, while no pathology was observed in sections from CG and SG rats, irregularity in gray matter morphology, increased vacuolization, and white matter infiltration into gray matter were significant findings in sections from ELMAG rats. Shrunken cytoplasm and dark staining in some gray matter neurons and intracytoplasmic vacuoles in part of the cytoplasm were also prominent.

Analysis of TUNEL-stained spinal cord sections from ELMAG rats exposed to the effects of 900-MHz EMF throughout adolescence revealed higher apoptotic indexes for both spinal cord glia cells and neurons compared to the other two groups. This shows that exposure to 900-MHz EMF applied throughout the adolescent period leads to apoptosis in both glia cells and neurons in the rat spinal cord. Previous studies have investigated apoptotic changes in other tissues (such as the testis and heart) that may develop as a result of exposure to 900-MHz EMF during adolescence (Hanci et al., 2013; Türedi et al., 2015). However, this study represents the first report of apoptotic changes occurring in the spinal cords of rats exposed to 900-MHz EMF in the stages of adolescence and the determination of those changes using the apoptotic index.

According to our results, MDA levels increased in the spinal cord of the ELMAG rats compared to those of the CG rats. It is therefore probable that the increase in MDA levels suggests injury caused by 900-MHz EMF in the spinal cords of the ELMAG rats. Another finding of this study is the decreases in SOD and GSH levels in ELMAG may be interpreted as resulting from the activity of antioxidant systems protecting the organism against oxidative injury occurring due to EMF (Ray and Husain, 2002; Consoles et al., 2012). GSH is an antioxidant found in mammal cells and that plays a role in the removal of free oxygen species (ROS). A decrease in GSH can therefore result in oxidative injury by leading to impairment of elimination of ROS forming in neurons (Övey and Naziroğlu, 2015). GSH and N-acetylcysteine (NAC), also a thiol-containing molecules, can exhibit a neuroprotective effect against oxidative stress (Naziroğlu et al., 2013). In other words, damage to neuronal tissue may occur in association with an increase in

oxidation products or through impairment of the functioning of antioxidant mechanisms.

In one study in which 2.45 GHz EMF was applied to rats in the pre- and postnatal periods, EMF was reported to cause a decrease in SOD levels in kidney tissue (Kuybulu et al., 2016). Koyu et al. (2009) reported a decrease in SOD and glutathione peroxidase (GSH-Px) in rat liver tissue in a group exposed to 900 MHz EMF. They also reported that SOD and GSH-Px levels in the EMF-exposed group returned to close to those of the control group following administration of the antioxidant caffeic acid phenethyl ester (CAPE). Another study reported that exposure of human lens epithelial cells to 1.8 GHz EMF led to a decrease in SOD1, SOD2, CAT and GSH-Px1 gene and protein expressions in the EMF group (Ni et al., 2013).

When the biochemical data are evaluated as a whole, significant differences were present between ELMAG and CG in terms of all biochemical parameters with the exception of CAT, while there were no significant differences between SG and ELMAG. We think that this may derive from the SG group, which may also be regarded as the true control group, also being exposed to cage-related stress. On the basis of the histopathological changes and increase in neuronal and glial apoptosis being more pronounced in the ELMAG group, we think that exposure to EMF led to additional cage-related stress in ELMAG and also SG. This shows that the stress to which the organism was exposed in SG was capable of compensation by the organism's antioxidant systems, but that this compensation was inadequate in ELMAG, suggesting that this insufficient compensation derived from structural and biochemical changes occurring in the spinal cords of ELMAG rats. It may therefore be concluded that EMF can result in oxidative injury in tissues both by increasing lipid peroxidation and by causing changes in antioxidant defense mechanisms (Ozguner et al., 2005; Ilhan et al., 2004; Meral et al., 2007; Hanci et al., 2015). Additionally, antioxidant mechanisms compromised under the effect of EMF exposure may have been unable to eliminate ROS forming both through EMF and through intrinsic mechanisms. Apoptosis and histopathological changes that increased significantly in ELMAG compared to SG may have occurred due to oxidative stress caused by exposure to EMF not only injuring the membrane lipids in the cell, but also DNA and proteins. The fact that biochemical parameters caused by oxidative stress in DNA, proteins and other cellular structures were not evaluated is a limitation of this study. This also makes it more difficult to account for the significant histological injury occurring between ELMAG and SG.

There are also studies in the literature reporting that EMF has no pathological effect on nervous system tissues. For example, Dogan et al. (2012) reported no change in apoptotic cells numbers of oxidative stress markers in brain tissues of rats exposed to EMF by mobile phone for 40 min daily over 20 days. Another study reported no significant change in neuron numbers in the hippocampus of the pups of pregnant rats exposed to the effect of EMF using cell phones (Ragbetli et al., 2009). The injury occurring in neuronal tissue in our study in contrast to these studies may be due to our employing a longer exposure time than Dogan et al. (2012) and Ragbetli et al. (2009). Another reason may be that the source of EMF used by us was different to that in those other studies. We used an oscillator as a source of EMF, while those other studies employed mobile phones. The oscillator used as a source of EMF in this study established continuous EMF, while there is a possibility that the EMF emitted by mobile phones can change.

In conclusion, our findings indicate that exposure to the effect of 900-MHz EMF (continuous, 1-h a day) from the beginning to the end of adolescence leads to impairment of normal morphological structures, to increased apoptosis in both neurons and glia cells

and to changes in tissue biochemical parameters (increased tissue MDA levels and decreased SOD and GSH levels in ELMAG) in the spinal cords of Sprague Dawley rats.

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